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(54) Method for Increasing gene expression.

(57) Disclosed are respiratory-deficient yeast except *Saccharomyces cerevisiae* AH22R⁻, said respiratory-deficient yeast being transformed with a DNA containing a gene encoding a protein foreign to yeast and a method for preparing a protein foreign to yeast described above, which comprises culturing the yeast, and producing and accumulating the protein in a culture. The respiratory-deficient yeast of the present invention can produce a higher amount of protein than its parent strain.

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METHOD FOR INCREASING GENE EXPRESSION

BACKGROUND OF THE INVENTION

The present invention relates to yeast for enhancing expression of a gene and a method for preparing a protein thereby.

Many studies have recently been conducted for producing useful proteins by yeast, using genetic engineering techniques. The reason is that expression of some genes which had been impossible in a prokaryote such as *Escherichia coli* became possible in yeast, a eukaryote. One example thereof is the expression of hepatitis B virus surface antigen having immunogenicity. When proteins having many disulfide bonds such as human lysozyme are produced, inactive types of human lysozyme are expressed intracellularly or secreted in expression using *Escherichia coli* or *Bacillus subtilis*. However, an active type of human lysozyme is expressed and secreted by yeast [K. Yoshimura et al., *Biochem. Biophys. Res. Commun.* 145, 712 (1987)].

However, the amounts of foreign genes expressed by yeast are generally lower than those expressed by *Escherichia coli*. Therefore, it is very important for industrial production of foreign proteins to increase the gene expression in yeast.

SUMMARY OF THE INVENTION

The present inventors have discovered that *Saccharomyces cerevisiae* AH22R⁻ strain (IFO 10134, FRI FERM BP-804) provides higher gene expression than other yeasts, and further that this strain is surprisingly respiratory-deficient. As a result of further studies of other yeasts, the present inventors have discovered that respiratory-deficient strains (ρ^-) of yeast (recombinant) transformed with expression plasmids of genes provide higher gene expression than their parent strains (ρ^+), namely that the gene expression is increased by rendering the yeast respiratory-deficient.

Yeast is able to grow under both anaerobic and aerobic conditions. ATP required for growth of yeast is acquired by a glycolysis system in cytoplasm under anaerobic conditions and by oxidative phosphorylation in mitochondria under aerobic conditions. Therefore, the respiratory-deficient strains (ρ^-) can be obtained by deleting a part or whole of mitochondria DNA [Kobo no Kaibo (Anatomy of yeast), edited by Naohiko Yanagishima, Taiji Oshima and Masako Osumi, Kodansha Scientific, p.137-147, 1981]. The respiratory-deficient strain where whole of the mitochondria DNA is deleted is sometimes represented by ρ^0 . In this specification, however, ρ^- and ρ^0 are totally represented by ρ^- .

In accordance with the present invention, there are provided respiratory-deficient yeast except *Saccharomyces cerevisiae* AH22R⁻, said respiratory-deficient yeast being transformed with a DNA containing a gene for foreign protein to yeast, and a method for preparing a foreign protein, which comprises culturing the yeast, accumulating the protein in a culture, and collecting the protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagram showing a process for synthesizing the synthetic human lysozyme gene by collection and ligation of oligonucleotides;

Fig. 2 shows a DNA sequence of the TaqI-XhoI fragment of the human lysozyme gene;

Fig. 3 shows a DNA sequence encoding the modified type of egg white lysozyme signal peptide;

Figs. 4 to 6 show schemes for the construction of the human lysozyme secretion plasmids used in the present invention;

Fig. 7 shows a scheme for the construction of the human EGF secretion plasmid used in the present invention; and

Fig. 8 shows a DNA sequence of the multi-linker used in the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The yeast strains for use in the present invention include, for example, *Saccharomyces cerevisiae* K33-

7B (a leu2 his pho80 pho8), *Saccharomyces cerevisiae* NA87-11A (α leu2 his3 trp1 pho3 pho5), *Saccharomyces cerevisiae* NA74-3A (a leu2 his4 pho9 can1), *Saccharomyces cerevisiae* NAX-50D (a leu2 his4 ura3 lys1 can1) and the like. *Saccharomyces cerevisiae* K33-7B described above was prepared by crossing *Saccharomyces cerevisiae* NA79-10C [Y. Kaneko et al., Mol. Cell. Biol. 5, 248 (1985)] with *Saccharomyces cerevisiae* AH22R⁻ [A. Miyanohara et al., Proc. Natl. Acad. Sci. U.S.A. 80, 1 (1983)]. *Saccharomyces cerevisiae* NA87-11A is described in Mol. Cell. Biol. 4, 771 (1984). *Saccharomyces cerevisiae* NAX-50D which was obtained by crossing NA74-3A with AX66-1B (α leu2 ura3 lys1 pho3) is a laboratory stock strain of the Institute for Fermentation (IFO), Osaka, Japan, and can be supplied from the Institute.

Any promoter for gene expression can be used as long as it functions in the yeast, which includes, for example, a promoter of glyceraldehyde-3-phosphate dehydrogenase gene (GLD), a promoter of repressive acid phosphatase gene (PHO5), a promoter of uridine-galactose diphosphate-4-epimerase gene (Gal 10), a promoter of galactokinase gene (Gal 1), a promoter of phosphoglycerate kinase gene (PGK), a promoter of alcohol dehydrogenase gene (ADH), a promoter of invertase gene (SUC2), a promoter for histidinol phosphate aminotransferase (HIS5), a promoter of α -factor gene and the like.

The gene product expressed by the yeast in the present invention includes animal enzymes, growth factors, hormones, lymphokines, viral proteins and the like. Examples include human lysozyme, protein disulfide isomerase (PDI), protein kinase C, human EGF (epidermal growth factor), basic FGF, nerve growth factor, growth hormone, insulin, interferon α , interferon β , interferon γ , interleukin 2, hepatitis B virus surface antigen, HTLV-I gag protein, lymphotoxin and the 5 like. Genes of molds or bacteria may also be used.

By ligating a DNA coding for a signal peptide upstream from the gene, the expression and secretion may be performed. Any signal peptide can be used as long as it functions in the yeast. Examples include egg white lysozyme and its modified type, human lysozyme, glucoamylase, α -factor, killer factor and the like. The secretion efficiency may be further enhanced by inserting a region coding for a propeptide between a signal peptide-encoding region and a gene to be expressed.

Any vector can be used as long as it functions in the yeast. Examples of the vector include pSH19 [S. Harashima et al., Mol. Cell. Biol. 4, 771 (1984)], pSH19-1 (European Patent Unexamined Publication No. EP-A-0235430) and the like. An expression vector can be obtained by inserting a promoter into the vector.

The plasmids for expression of the gene can be obtained by inserting the gene downstream from the promoter in the expression vector described above. In many cases, the gene expression can be increased by inserting a terminator downstream from the gene. The terminator includes those of phosphoglycerate kinase gene (PGK), FLP gene of 2 μ DNA, invertase gene (SUC2) and the like.

Methods for constructing the expression plasmid in the present invention are known, one of which is described, for example, in Molecular Cloning (1982), Cold Spring Harbor Laboratory.

Any expression plasmid can be used as long as it expresses the gene in the yeast, producing the gene product either inside cells (intracellular expression) or outside cells (expression and secretion). The expression and secretion plasmid includes expression and secretion plasmids pGEL125 [K. Yoshimura et al., Biochem. Biophys. Res. Commun. 145, 712 (1987)], pGFL735, pGFL725T, pTFL710T, pTFL771T, pTFL780T for human lysozyme, an expression and secretion plasmid pGFE213 for human EGF and the like.

Using the expression plasmid obtained as described above, the yeast is transformed. The transformation methods, which are known per se, include for example the lithium method [Ito et al., J. Bacteriol. 153, 163 (1983)], the protoplast method [Hinnen et al., Proc. Natl. Acad. Sci. U.S.A. 75, 1927 (1978)] and the like. Thus, the yeast (recombinant) having the expression plasmid is obtained.

Methods for obtaining the respiratory-deficient strain (ρ^-) from the yeast having respiratory activity (parent strains ρ^+), which are known per se, include for example the method described in Laboratory Course Manual for Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1986. That is, the respiratory-deficient strain can be easily obtained by culturing the parent strain in a medium containing ethidium bromide, and then isolating the strain which can grow in a medium containing glucose as a carbon source, but cannot grow in a medium containing glycerol. Although the frequency is low, the respiratory-deficient strain can also be obtained by single colony isolation from the parent strain. When the parent strain is a transformant (recombinant) having an expression plasmid, the desired recombinant by which gene expression is increased can be directly obtained by isolating its respiratory-deficient strain. When the parent strain carries no expression plasmid, the desired recombinant can be obtained by isolating its respiratory-deficient strain and then introducing an expression plasmid therein.

The transformant (recombinant) thus obtained is cultured by methods which are known per se.

Examples of the medium include Burkholder minimum medium [K. L. Bostian et al. Amper. J. Bot. 30, 206 (1943)], its modified medium [A. Toh-E et al., J. Bacteriol. 113, 727 (1973)] or lower phosphate medium

[A. Toh-e et al., 113, 727 (1973)]. The cultivation is generally conducted at 15 to 40° C, preferably 24 to 37° C for 10 to 168 hours, preferably 72 to 144 hours with or without shaking, aeration or agitation if necessary.

After the completion of the cultivation, the supernatant is separated from the cells by methods which are known per se. For example, to obtain human lysozyme or human EGF remaining within the cells, the cells are disrupted by conventional methods such as a disruption using ultrasonic treatment or French press, mechanical disruption such as crushing and disruption by a cell wall lytic enzyme. Further, if necessary, the human lysozyme thus produced may be extracted by addition of a surfactant such as Triton-X100 or deoxycholate. The human lysozyme or human EGF contained in the supernatant or the extract thus obtained is purified by conventional protein purification methods such as salting out, isoelectric point precipitation, gel filtration, ion-exchange chromatography and high performance liquid chromatography (HPLC, FPLC, etc.) to obtain the desired human lysozyme or human EGF.

The activity of human lysozyme obtained as described above can be assayed by the method described in Yochimura et al., Biochem. Biophys. Res. Commun. 145, 712 (1987), in which decrease in absorbance of Micrococcus luteus cells is used as an index. The human EGF can be determined by the radioimmunoassay supplied by Amersham Inc., the fibroblast receptor assay [Proc. Natl. Acad. Sci. U.S.A. 72, 1371 (1975)], etc.

Gene products other than human lysozyme and human EGF can also be separated and purified by known methods.

The gene expression by the respiratory-deficient yeast is generally increased about 1.5-fold to 10-fold as compared with the parent cell(ρ^+).

The present invention will hereinafter be described in detail with the following Reference Examples and Examples. It is understood of course that these Reference Examples and Examples are not intended to limit the scope of the invention.

Reference Example 1

Construction of Human Lysozyme Secretory Plasmid pGFL735

After 5 μ g of Escherichia coli vector pBR322 was reacted with 1.5 units of restriction enzyme BalI in 40 μ l of a reaction solution [10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol] at 37° C for 5 hours, the reaction mixture was treated with phenol and a DNA fragment was precipitated with ethanol in accordance with conventional methods. To this DNA fragment, 50 ng of a phosphorylated XhoI linker d-[pCCTCGAGG] (New England Biolabs) was added and they were ligated with each other by T4 DNA ligase according to a conventional method.

An Escherichia coli DH1 strain was transformed with this reaction solution and a plasmid was extracted from the ampicillin-resistant and tetracycline-resistant transformant thus obtained, according to the alkaline extraction method [H. C. Birnboim and J. Doly, Nucl. Acids Res., 7, 1513 (1979)] to obtain a plasmid pBR322X having an XhoI site in place of the BalI site.

In the report of Ikehara et al., [Chem. Pharm. Bull. 34, 2202 (1986)], the human lysozyme gene is prepared, for example, from the 52 oligonucleotide blocks shown in Table 1.

Table 1

	Upper Strand No.		Lower Strand No.	
5	U1	TCGAGATGAAGGTTT	L26	TCGAGCTATTAAAC
	U2	TTGAGAGATGCGAAT	L25	ACCACAACCTTGAAC
	U3	TAGCCAGAACTTTGAAG	L24	GTATTGTCTGACATC
	U4	AGATTGGGTATGGAC	L23	TCTATTTTGGCATCT
	U5	GGCTACCGTGGTATT	L22	GTTTCTCCAAGCGAC
10	U6	TCTTTAGCCAAGTGG	L21	CCAGGCTCTAATACCCTG
	U7	ATGTGTCTTGCTAAG	L20	TGGGTCACGGACAAC
	U8	TGGGAATCCGGCTATAAC	L19	TCTCTTAGCGCAGGC
	U9	ACTAGAGCTACCAAT	L18	AACAGCTCAGCAAT
	U10	TACAACGCTGGCGAC	L17	GTTGTCCTGAAGC
15	U11	CGTTCTACAGACTATGG	L16	AAAGCTGAGCAAGAT
	U12	TATTTTCCAAATTAAC	L15	AAGTGACAGGCGTTGAC
	U13	CTAGATATTGGTG	L14	GGCACCTGGAGTCTTGC
	U14	TAACGATGGCAAGACTC	L13	CATCGTTACACCAATAT
	U15	CAGGTGCCGTCACGCGC	L12	CTAGAGTTAATTTGG
20	U16	TGTCACCTTATCTTGC	L11	AAAATACCATAGTCTGT
	U17	TCAGCTTTGCTTCAG	L10	AGAACGGTCGCCAGC
	U18	GACAACATTGCTGAT	L9	GTTGTAATTGGTAGC
	U19	GCTGTTGCCTGCGCT	L8	TCTAGTGTTATAGCCG
	U20	AAGAGAGTTGTCCGT	L7	GATTCCCATTAGCAAG
25	U21	GACCCACAGGGTATT	L6	ACACATCCAGTTGGC
	U22	AGAGCCTGGGTGCGCT	L5	TAAAGAAATACCACG
	U23	TGGAGAAACAGATGC	L4	GTAGCCGTCCATACC
	U24	CAAAATAGAGATGTC	L3	CAATCTCTTCAAAGT
	U25	AGACAATACGTTCAAGG	L2	TCTGGCTAATTTCGCATC
30	U26	TTGTGGTGTTTAATAGC	L1	TCTCAAAAACCTTCATC

In Table 1, CGAGAGATGCGAAT was synthesized as U2-taq in place of U2, and TCTGGCTAATTCG-CATCTCT was synthesized as L2-taq in place of L2, according to the report of Ikehara et al. Then, using fragments U2-taq, U3 to U26, L2-taq and L3 to L26, the respective hybrids of oligonucleotide blocks were formed according to the report of Ikehara et al. (Fig. 1). After each of these groups was ligated by T4 DNA ligase, both 5'-termini were enzymatically phosphorylated.

2.6 µg of the plasmid pBR322X was reacted with 6 units of restriction enzyme XhoI and 6 units of restriction enzyme ClaI in 35 µl of a reaction solution [33 mM acetate buffer, pH 7.9, 66mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 0.01% BSA] at 37 °C for 1 hour. Then, the solution was deproteinized with phenol and precipitated with cold ethanol. This DNA fragment (200 ng) was mixed with 100 ng of the human lysozyme gene fragment prepared above and allowed to ligate with each other in 10 µl of a reaction solution [66 mM Tris-HCl (pH 7.6), 10 mM ATP, 10 mM spermidine, 100 mM MgCl₂, 150 mM DTT, 2 mg/ml BSA, 5 units of T4 DNA ligase] at 14 °C overnight. Using this reaction solution, an *Escherichia coli* DH1 strain was transformed according to the method of Cohen et al. [Proc. Natl. Acad. Sci. U.S.A. 69, 2110 (1972)]. A plasmid was isolated from the transformant thus obtained according to the alkaline extraction method previously mentioned. Their molecular weight and cleavage pattern by restriction enzymes were examined and pLYS221 in which the human lysozyme gene fragment was inserted was obtained. The ECoRI-XhoI fragment of pLYS221 was isolated and its base sequence was determined in accordance with the dideoxynucleotide synthetic chain termination method. As a result, the TaqI-XhoI fragment of human lysozyme gene was obtained as shown in Fig. 2 exactly as assumed.

This sequence codes for the Glu-4 to the Val-130 of the amino acid sequence of human lysozyme.

The Leu-4, the Ile-6 and the Val-8 of the known amino acid sequence of the egg white lysozyme signal peptide [A. Jung et al., Proc. Natl. Acad. Sci. 77, 5759 (1980)] were replaced with Phe, Leu and Ala, respectively. The nucleotide sequence was determined in consideration of the following points for high expression:

- (1) Codons which are frequently used in yeast are preferentially selected;
- (2) To enhance the expression, a sequence of the yeast PGK gene is used upstream from ATG; and

(3) Construction of a hybrid signal is possible.

The nucleotide sequence thus synthesized is shown in Fig. 3. There are provided an XhoI site at the 5'-terminus and a TaqI site at the 3'-terminus containing the human lysozyme-encoding region. The whole sequence consists of 8 oligonucleotide blocks (#1-#8), which were prepared by the phosphoramidite method [M. H. Caruthers et al., Tetrahedron Letters 22, 1859 (1981)].

The oligonucleotide blocks #2 to #7 were first mixed with each other in 10 μ l (5 μ g) portions, to which were further added 20 μ l of a kinase buffer of a 10-fold concentration (0.5 M Tris-HCl, 0.1 M MgCl₂, 0.1 M mercaptoethanol, pH 7.6), 20 μ l of 10 mM ATP, 20 μ l (50 u) of T4 polynucleotide kinase (Takara Shuzo Inc.) and 80 μ l of distilled water. The mixture was then reacted at 37°C for 2 hours and thereafter treated at 65°C for 20 minutes to stop the reaction. To this reaction mixture were added the oligonucleotide blocks #1 and #8 in 10 μ l (5 μ g) portions. Further, 10 μ l of T4 ligase (NEB Inc.) was added thereto and the mixture was reacted at 14°C overnight. The resulting reaction mixture was subjected to 10% polyacrylamide electrophoresis. A 76 bp fragment was cut out and extracted from the gel by electroelution. This fragment was dissolved in 45 μ l of distilled water, to which were added 6 μ l of the kinase buffer of a 10-fold concentration previously mentioned, 6 μ l of 10 mM ATP and 2 μ l (5 u) of T4 polynucleotide kinase previously mentioned. The mixture was reacted at 37°C for 1 hour and then stored at -20°C.

In Fig. 3, there is adopted a single letter expression for amino acids (Rule Confirmed by IUPAC-IUB Biochemistry Nomenclature).

Example:

A: Alanine

B: Aspartic acid or asparagine

C: Cysteine

D: Aspartic acid

E: Glutamic acid

F: Phenylalanine

G: Glycine

H: Histidine

I: Isoleucine

K: Lysine

L: Leucine

M: Methionine

N: Asparagine

P: Proline

O: Glutamine

R: Arginine

S: Serine

T: Threonine

V: Valine

W: Tryptophan

Y: Tyrosine

Z: Glutamic acid or glutamine

X: Unknown or other amino acids

The plasmid pLYS221 (236 μ g) was treated with 120 u of EcoRI (Nippon Gene Inc.) and 120 u of XhoI (Nippon Gene Inc.) at 37°C for 2 hours to cut out a fragment of the human lysozyme-encoding region. This fragment was further treated with 26 u of TaqI (Nippon Gene Inc.) at 65°C for 1 hour to cut out a fragment of the human lysozyme-encoding region from which a part of the N-terminal portion was deleted.

About 1 μ g of this fragment was mixed with 0.5 μ g of a DNA fragment coding for the signal sequence described above and the mixture was reacted in the presence of 800 u of T4 ligase previously mentioned at 16°C for 16 hours, followed by treatment with XhoI (42 u).

The obtained XhoI fragment (10 ng) was mixed with 1 ng of a fragment obtained by treating yeast expression vector pGLD906-1 (Japanese Patent Unexamined Publication No. 43991/1986) with XhoI, and both were ligated with each other in the presence of T4 ligase.

Escherichia coli DH1 was transformed with the resulting reaction mixture by the method described above to obtain a number of plasmids where the signal sequence-encoding region and the human lysozyme gene were inserted downstream from the GLD promoter in the same direction as that of the promoter. One of such plasmids was named pGFL735 and used in the following experiments (see Fig. 4).

Reference Example 2Construction of Human Lysozyme Expression Plasmid pPFL725T (Fig. 5)

5 A 0.28 kb *AhaIII*-*Sall* fragment containing a PGK terminator was isolated from a modified HBsAg P31 expression plasmid PGLD P31-RCT (European Patent Unexamined Publication No. 0235430). An *Sall* linker pGGTCGACC was ligated with this fragment by T4 DNA ligase, followed by treatment with *Sall*. The 0.28 kb *Sall* fragment and the *Sall* linker not ligated with the fragment were separated by agarose gel electrophoresis and the 0.28 kb *Sall* fragment was obtained from agarose gel by the isolating method of DNA using DEAE cellulose paper [G. Winberg and M. L. Hammarström, *Nucl. Acids Res.* 8, 253 (1980)].

10 A human lysozyme expression plasmid pGEL125 using the egg white lysozyme signal peptide [K. Yoshimura et al., *Biochem. Biophys. Res. Commun.* 145, 712 (1987)] was cleaved with *Bam*HI and *Xho*I, from which a 1.5 kb *Bam*HI-*Xho*I fragment containing the GLD promoter and the egg white lysozyme signal peptide-encoding region and the human lysozyme-encoding region, and the residual 8.4 kb *Bam*HI-*Xho*I fragment were separated by agarose gel electrophoresis. Then, each of them was isolated.

15 The 0.28 kb *Sall* fragment containing the PGK terminator was ligated by T4 DNA ligase to the 3'-terminus (*Xho*I site) of the above-described 1.5 kb *Bam*HI-*Xho*I fragment containing the GLP promoter, the egg white lysozyme signal peptide-encoding region and the human lysozyme-encoding region, and then subjected to agarose gel electrophoresis to isolate a 1.8 kb *Bam*HI-*Sall* fragment.

20 The 1.8 kb *Bam*HI-*Sall* fragment thus obtained was ligated with the 8.4 kb *Bam*HI-*Xho*I fragment by T4 DNA ligase. Using the resulting fragment, *Escherichia coli* DH1 was transformed. A plasmid was prepared from the ampicillin-resistant transformant, and was named pGEL125T.

25 From the human lysozyme expression plasmid pGFL735 obtained in Reference Example 1, a 0.5 kb *Xho*I fragment coding for the modified signal peptide and human lysozyme was isolated. On the other hand, an expression vector pPHO17-1 having the PHO-5 promoter (European Patent Unexamined Publication No. 0235430) was cleaved with *Xho*I and then ligated with the 0.5 kb *Xho*I fragment, followed by transformation of *Escherichia coli*. A plasmid was prepared from the ampicillin-resistant transformant thus obtained, and was named pPFL725.

30 Each of plasmids pGEL125T and pPFL725 obtained as described above had *Xba*I cleavage sites upstream from the promoter and in the human lysozyme-encoding region. Both plasmids were cleaved with *Xba*I, and the 1.7 kb *Xba*I fragment from pPFL725 was ligated with the 7.9 kb *Xba*I fragment from pGEL125T by T4 DNA ligase, followed by transformation of *Escherichia coli* DH1. A plasmid was isolated from the ampicillin-resistant transformant thus obtained, and was named pPFL725T.

Reference Example 3Construction of Human Lysozyme Expression Plasmid pTFL710T (Fig. 6)

40 A 2.3 kb *Xba*I fragment and a 7.9 kb *Xba*I fragment were isolated from the human lysozyme expression plasmid pGFL 735 obtained in Reference Example 1 and from the plasmid pGFL125T obtained in Reference Example 2, respectively. Both fragments were then ligated with each other by T4 DNA ligase, followed by transformation of *Escherichia coli* DH1. A plasmid was isolated from the ampicillin-resistant transformant, and was named pGFL735T.

45 An *Eco*RI-*Xho*I adapter was ligated by T4 DNA ligase with a 0.7 kb *Bgl*II-*Eco*RI fragment containing the Gal 10 promoter obtained from plasmid p286, which was obtained by replacing a 275 bp *Sall*-*Bam*HI fragment located upstream from the Gal 10 promoter of a plasmid pBM150 [*Mol. Cell. Biol.* 4, 1440 (1984)] with a multi-linker of about 40 bp having restriction enzyme sites such as *Sac*II, *Bam*HI, *Sall* and *Bgl*II (Fig. 8). The resulting product was then treated with *Bgl*II and *Xho*I, and a 0.7 kb *Bgl*II-*Xho*I fragment was isolated by agarose gel electrophoresis.

50 On the other hand, the 1.1 kb *Bam*HI-*Xho*I fragment containing the GLD promoter of the plasmid pGFL735T was removed, and the *Bgl*II-*Xho*I fragment containing the Gal 10 promoter was inserted therein, followed by transformation of *Escherichia coli* DH1. A plasmid was isolated from the ampicillin-resistant transformant thus obtained, and was named pTFL710T.

Reference Example 4Construction of Human EGF Expression Plasmid pGFE213 (Fig. 7)

The oligonucleotide block #9 and the oligonucleotide block #10 shown in Fig. 7 were chemically synthesized in place of the oligonucleotide blocks #5 and #7 and the oligonucleotide blocks #6 and #8 shown in Fig. 3 of Reference Example 1, respectively, and ligated with the oligonucleotide blocks #1 to #4 by T4 DNA ligase to prepare a 76 bp XhoI-HinfI fragment coding for a modified type of signal peptide and the N-terminal region of human EGF. A 0.16 kb HinfI-PstI fragment coding for human EGF from which a portion of the N-terminus was removed was isolated from a plasmid pTB370 [Taniyama et al., J. Takeda Res. Lab. 45, 136 1986], Japanese Patent Unexamined Publication 88881/1986], and to the 5'-terminus thereof was ligated the 76 bp XhoI-HinfI fragment described above by T4 DNA ligase, followed by treatment with XhoI and PstI to isolate a 0.24 kb XhoI-PstI fragment. A PstI-SmaI adapter was ligated with this fragment by T4 DNA ligase, followed by treatment with XhoI and SmaI to isolate a 0.24 kb XhoI-SmaI fragment coding for the modified type signal peptide and human EGF.

Plasmid pGFL735Sm obtained by modifying the XhoI site located downstream from the human lysozyme-encoding region of the plasmid pGFL735 obtained in Reference Example 1 to an SmaI site was cleaved with XhoI and SmaI, and a DNA fragment coding for the modified type of signal peptide and human lysozyme was removed. In place of the removed DNA fragment, the 0.24 kb XhoI-SmaI fragment described above was inserted to obtain plasmid pGFE101.

A 0.28 kb AhaIII-XhoI fragment containing the PGK terminator was isolated from the plasmid pGLD P31-RcT previously mentioned and inserted in the SmaI-SalI site of a plasmid pSP64 (Riboprobe Inc. U.S.A.) to obtain a plasmid pSP64-T (PGK). An EcoRI-PstI fragment containing the PGK terminator was isolated from this plasmid, and the PstI-SmaI adapter and a SmaI-EcoRI adapter were added thereto, followed by insertion of the resulting product into the SmaI site of the plasmid pGFE101 described above to obtain a human EGF expression plasmid pGFE213.

Example 1Preparation of Transformants

Using the human lysozyme expression plasmid pGFL735 obtained in Reference Example 1, *Saccharomyces cerevisiae* K33-7B (a leu2 his pho80 pho8), *Saccharomyces cerevisiae* NA87-11A (α leu2 his3 trp1 pho3 pho5) and *Saccharomyces cerevisiae* NA74-3A (a leu2 his4 pho9 can1) were transformed by the lithium method previously mentioned to obtain transformants *Saccharomyces cerevisiae* K33-7B/pGFL735, *Saccharomyces cerevisiae* NA87-11A/pGFL735 and *Saccharomyces cerevisiae* NA74-3A/pGFL735, respectively.

Using the human lysozyme expression plasmid pPFL725 obtained in Reference Example 2, the human lysozyme expression plasmid pTFL710T obtained in Reference Example 3 and the human FEG expression plasmid pGFE213 obtained in Reference Example 4, *Saccharomyces cerevisiae* NA74-3A was transformed by the lithium method to obtain transformants *Saccharomyces cerevisiae* NA74-3A/pPFL725T, *Saccharomyces cerevisiae* NA74-3A/pTFL710T and *Saccharomyces cerevisiae* NA74-3A/pGFE213, respectively.

Further, using the human lysozyme expression plasmid pTFL710T obtained in Reference Example 3, a transformant of *Saccharomyces cerevisiae* NAX-50D, *Saccharomyces cerevisiae* NAX-50D/pTFL710T, was prepared by the lithium method.

Example 2Preparation of Respiratory-Deficient Strains of Transformants (Recombinants)

Using ethidium bromide in accordance with the method described in "Laboratory Course Manual for Methods in Yeast Genetics (Cold Spring Harbor Laboratory, 1986)", respiratory-deficient strains *Sac-*

charomyces cerevisiae K33-7B(ρ^-)/pGFL735, Saccharomyces cerevisiae NA87-11A (ρ^-)/pGFL735, Saccharomyces cerevisiae NA74-3A (ρ^-) pGFL735, Saccharomyces cerevisiae NA74-3A (ρ^-)/pPFL725T, Saccharomyces cerevisiae NA74-3A (ρ^-)/pTFL710T, Saccharomyces cerevisiae NAX-50D (ρ^-) /pTFL710T and Saccharomyces cerevisiae NA74-3A (ρ^-)/pGFE213 were prepared from the transformants (recombinants),
 5 Saccharomyces cerevisiae K33-7B/pGFL735, Saccharomyces cerevisiae NA87-11A/pGFL735, Saccharomyces cerevisiae NA74-3A/pGFL735, Saccharomyces cerevisiae NA74- 3A/pPFL725T, Saccharomyces cerevisiae NA74-3A/pTFL710T, Saccharomyces cerevisiae NAX50D/pTFL710T and Saccharomyces cerevisiae NA74-3A/pGFE213, respectively, which were obtained in Example 1.

Example 3

Preparation of Transformant (Recombinant) of Respiratory-Deficient Strain

From Saccharomyces cerevisiae NA74-3A, its respiratory-deficient strain (ρ^-) was separated by the method shown in Example 2, and transformed according to the lithium method with the human lysozyme expression plasmid pTFL710T prepared in Reference Example 3 to obtain a transformant Saccharomyces cerevisiae NA74-3A (ρ^-)/pTFL710T.

Example 4

Secretory Production of Human Lysozyme Using Plasmid pGFL 735

The recombinants obtained in Example 1, Saccharomyces cerevisiae K33-7B/pGFL735, Saccharomyces cerevisiae NA87-11A/pGFL735 and Saccharomyces cerevisiae NA74-3A/pGFL735, and their respiratory-deficient strains obtained in Example 2, Saccharomyces cerevisiae K33-7B (ρ^-)/pGFL735, Saccharomyces cerevisiae NA87-11A (ρ^-)/pGFL735 and Saccharomyces cerevisiae NA74-3A (ρ^-)/pGFL735 were inoculated
 30 into modified Burkholder medium containing 8.9% sucrose, 1.1% glucose and 0.044% KH_2PO_4 [A. Toh-E et al., J. Bacteriol. 113, 727 (1973)], and cultured at 30°C for 3 days with shaking. 1 ml of each of the culture was transferred to a test tube containing 4 ml of the same medium and cultured at 30°C for 1 day with shaking. Subsequently, 2 ml of the culture was transferred to a 200 ml Erlenmeyer flask containing 18 ml of
 35 the same medium and cultured at 30°C for 72 hours with shaking. The cultures thus obtained were centrifuged and the human lysozyme activity of the supernatants was measured by the method described in Yoshimura et al., Biochem. Biophys. Res. Commun. 145, 712 (1987) to determine the amounts of human lysozyme produced in the supernatants. The results are shown in Table 2.

Table 2

Recombinant	Cultivation time (hour)	Growth (Klett)	Human lysozyme (mg/L)
S.cerevisiae K33-7B/pGFL735	72	443	0.5
S.cerevisiae K33-7B(ρ^-)/pGFL735	72	432	3.3
S.cerevisiae NA87-11A/pGFL735	72	470	2.5
S.cerevisiae NA87-11A(ρ^-)/pGFL735	72	440	5.3
S.cerevisiae NA74-3A/pGFL735	72	520	1.4
S.cerevisiae NA74-3A(ρ^-)/pGFL735	72	490	6.0
S.cerevisiae NA74-3A/pPFL725T	120	540	3.3
S.cerevisiae NA74-3A(ρ^-)/pPFL725T	120	500	6.7
S.cerevisiae NA74-3A/pTFL710T	144	530	2.0
S.cerevisiae NA74-3A(ρ^-)/pTFL710T	144	505	18
S.cerevisiae NAX-50D/pTFL710T	96	405	0.9
S.cerevisiae NAX-50D(ρ^-)/pTFL710T	96	380	5.3

As apparent from Table 2, when the human lysozyme expression plasmid pGFL735 having the GLD promoter was used, the human lysozyme production by the respiratory-deficient strain (ρ^-) was 2 to 6 times higher than that by its parent strain (ρ^+) in all yeast strains.

Example 5

Secretory Production of Human Lysozyme Using Plasmid pPFL725T

The recombinant *Saccharomyces cerevisiae* NA74-3A/pPFL725T obtained in Example 1 and its respiratory-deficient strain *Saccharomyces cerevisiae* NA74-3A(ρ^-)/pPFL725T obtained in Example 2 were inoculated into high phosphate medium [A. Toh-E et al., J. Bacteriol. 113, 727 (1973)] and cultured at 30 °C for 3 days with shaking. 1 ml of each of the culture was transferred to a test tube containing 4 ml of the same medium and cultured at 30 °C for 1 day with shaking. Subsequently, 2 ml of the culture was transferred to a 200 ml Erlenmeyer flask containing 18 ml of low phosphate medium [the modified Burkholder medium previously mentioned which contained 8.9% sucrose, 1.1% glucose and 0.003% KH_2PO_4] and cultured at 30 °C for 120 hours with shaking.

The human lysozyme activity of the supernatants of the cultures thus obtained was measured to determine the amounts of human lysozyme produced in the supernatants.

As a result, it was revealed that the respiratory-deficient strain (ρ^-) showed the human lysozyme production about 2 times higher than its parent strain (ρ^+), also when the human lysozyme expression plasmid pPFL725T having the PHO5 promoter was used (Table 2).

Example 6

Secretory Production of Human Lysozyme Using Plasmid pTFL710T

The recombinant *Saccharomyces cerevisiae* NA74-3A/pTFL710T obtained in Example 1 and its respiratory-deficient strain *Saccharomyces cerevisiae* NA74-3A(ρ^-)/pTFL710T obtained in Example 2 were inoculated into the modified Burkholder medium previously mentioned which contained 8.9% sucrose, 1.1% glucose and 0.044% KH_2PO_4 and cultured at 30 °C for 3 days with shaking. 1 ml of each of the culture was transferred to a test tube containing 4 ml of the same medium and cultured at 30 °C for 1 day with shaking. Subsequently, 2 ml of the culture was transferred to a 200 ml Erlenmeyer flask containing 18 ml of a galactose medium [the modified Burkholder medium previously mentioned which contained 8.9% sucrose, 5% galactose, 0.25% glucose and 0.044% KH_2PO_4] and cultured at 30 °C for 144 hours with shaking.

The human lysozyme activity of the supernatants of the cultures thus obtained was measured to determine the amounts of human lysozyme produced in the supernatants.

As a result, it was revealed that the respiratory-deficient strain (ρ^-) showed the human lysozyme production about 2 times higher than its parent strain (ρ^+), also when the human lysozyme expression plasmid pTFL710T having the Gal10 promoter was used (Table 2).

The recombinant *Saccharomyces cerevisiae* NA74-3A/pTFL710T obtained in Example 1 and the transformant of its respiratory-deficient strain, *Saccharomyces cerevisiae* NA74-3A(ρ^-)/pTFL710T, obtained in Example 3 were cultured in a similar manner. Similarly to the results described above, the human lysozyme production by *Saccharomyces cerevisiae* NA74-3A(ρ^-)/pTFL710T was higher than that by *Saccharomyces cerevisiae* NA74-3A/pTFL710T.

The transformant *Saccharomyces cerevisiae* NAX-50D/pTFL710T obtained in Example 1 and its respiratory-deficient strain *Saccharomyces cerevisiae* NAX-50D(ρ^-)/pTFL710T were cultured in a similar manner for 96 hours. Consequently, the latter produced human lysozyme in quantity about 6 times higher than the former (Table 2).

Example 7

Secretory Production of Human EGF Using Plasmid pGFE213

The recombinant *Saccharomyces cerevisiae* NA74-3A/pGFE213 obtained in Example 1 and its respiratory-deficient strain *Saccharomyces cerevisiae* NA74-3A(ρ^-)/pGFE213 obtained in Example 2 were cultured in the same manner as in Example 4. The cultures thus obtained were centrifuged and human EGF in the supernatants was determined by use of a kit for radioimmunoassay (Amersham Inc.). The results are shown in Table 3.

Table 3

10	Recombinant	Growth (Klett)	Human EGF (mg/L)
	<u>S. cerevisiae</u> NA74-3A/pGFE213	525	0.56
	<u>S. cerevisiae</u> NA74-3A(ρ^-)/pGFE213	475	0.85

As described above, the human EGF production by the respiratory deficient strain (ρ^-) was higher than that by its parent strain (ρ^+), also when the human EGF gene was used as a gene.

Reference Example 5

Construction of Human Lysozyme Expression Plasmid pTFL771T

The EcoRI-XhoI adapter was ligated by T4 DNA ligase with the 0.7 kb BglII-EcoRI fragment containing the Gal 10 promoter, which was shown in Reference Example 3. The resulting product was then treated with Sau3AI and XhoI, and subjected to agarose gel electrophoresis to isolate a 0.51 kb Sau3AI-XhoI fragment containing the Ga110 promoter.

On the other hand, the 1.1 kb BamHI-XhoI fragment containing the GLD promoter was removed from the plasmid pGFL735T, which was obtained in Reference Example 3, and the above-described 0.51 kb Sau3AI-XhoI fragment containing the Gal 10 promoter was ligated by T4 DNA ligase in place of the removed fragment. A plasmid was isolated from the transformant of *Escherichia coli* DH1, and was named pTFL771T.

Reference Example 6

Construction of Human Lysozyme Expression Plasmid pTFL780T

The 0.7 kb BamHI-XhoI fragment containing the Ga110 promoter was removed from the plasmid pTFL710 obtained in Reference Example 3, and the 0.51 kb Sau3AI-XhoI fragment obtained in Reference Example 5 was inserted in place of the removed fragment to obtain human lysozyme expression plasmid pTFL780T.

Example 8

Preparation of Transformants

Using the human lysozyme expression plasmid pTFL771T obtained in Reference Example 5 and the human lysozyme expression plasmid pTFL780T obtained in Reference Example 6, *Saccharomyces cerevisiae* NA74-3A and its respirator- deficient strain *Saccharomyces cerevisiae* NA74-3A (ρ^-) (Example 3) were transformed according to the lithium method to obtain transformants *Saccharomyces cerevisiae* NA74-3A/pTFL771T, *Saccharomyces cerevisiae* NA74-3A (ρ^-)/pTFL771T, *Saccharomyces cerevisiae* NA74-3A/pTFL780T and *Saccharomyces cerevisiae* NA74-3A (ρ^-)/pTFL780T, respectively.

Example 9Secretory Production of Human Lysozyme Using Plasmids pTFL771T and pTFL780T

The transformants obtained in Example 8 were cultured in a similar manner as in Example 6 and the amount of human lysozyme produced by each transformant was determined. As a result, in each case of pTFL771 and pTFL780T, the transformant of the respiratory-deficient strain produced more human lysozyme than did the transformant of its parent strain, as in Example 6.

Example 10Secretion of Human Lysozyme using α -Factor Promoter and Prepro Region

The DNA fragment containing the GLD promoter and signal peptide-coding region in the plasmid pGEL125 was substituted with a DNA fragment containing the promoter and prepro region of α -factor gene which was derived from p69A[Cell, 30, 933(1982)] to construct a plasmid PAAL410 for secretion of human lysozyme.

On the other hand, *Saccharomyces cerevisiae* NA74-3A was crossed with *Saccharomyces cerevisiae* DK-13D (α leu2 his3 trp1)[Mol. Cell. Biol., 4, 771 (1984)] to prepare *Saccharomyces cerevisiae* TA39 (α leu2 his can1), from which respiratory-deficient strain *Saccharomyces cerevisiae* TA39 (ρ^-) was obtained according to the method described in Example 2.

The above *Saccharomyces cerevisiae* TA39 and its respiratory-deficient strain *Saccharomyces cerevisiae* TA39 (ρ^-) were transformed with the above plasmid pAAL410 for secretion of human lysozyme by the lithium method to give *Saccharomyces cerevisiae* TA39/pAAL410 and *Saccharomyces cerevisiae* TA39(ρ^-)/pAAL410, respectively.

The transformants *Saccharomyces cerevisiae* TA39/pAAL410 and *Saccharomyces cerevisiae* TA39(ρ^-)-pAAL410 were cultivated in the same method as that of Example 4 and the obtained supernatant of the culture was subjected to an assay for human lysozyme activity to determine the yield of human lysozyme. The assay showed that *Saccharomyces cerevisiae* TA39/pAAL410 and *Saccharomyces cerevisiae* TA39(ρ^-)/pAAL410 produced 0.5 mg/l and 2.6 mg/l of human lysozyme, respectively. That is, the respiratory-deficient strain (latter) yielded human lysozyme five times as much as the parent strain (former).

The following microorganisms which are disclosed in the Examples and Reference Examples have been deposited in the Institute for Fermentation (IFO), Osaka, Japan and in the Fermentation Research Institute, Agency of Industrial Science and Technology (FRI), Japan under the Budapest Treaty.

Microorganism <i>Saccharomyces cerevisiae</i>	IFO	FRI(FERM)
1. NA74-3A	10430	BP-1947(P-9691)
2. NA74-3A(ρ^-)	10431	BP-1948(P-9692)
3. NA74-3A/pTFL710T	10432	BP-2090(P-9693)
4. NA74-3A(ρ^-)/pTFL710T	10433	BP-2091(P-9694)
5. K33-7B(ρ^-)	10457	BP-2092
6. NA87-11A(ρ^-)	10458	BP-2093
7. NA74-3A(ρ^-)/pPFL725T	10459	BP-2094
8. NA74-3A(ρ^-)/pGFE213	10460	BP-2095
9. NAX-50D(ρ^-)/pTFL710T	10461	BP-2096
10. AH22R $^-$ /pGFL735	10227	BP-1346
11. AH22R $^-$ /pGEL125	10211	BP-1345(P-8806)
12. AH22R $^-$ /pGLD P31-RcT	10206	BP-1059

Claims

1. Respiratory-deficient yeast excluding Saccharomyces cerevisiae AH22R⁻, said respiratory-deficient yeast being transformed with a DNA containing a gene encoding a protein foreign to yeast.
- 5 2. Yeast as claimed in claim 1, wherein the gene is a human lysozyme gene or a human EGF gene.
3. Yeast as claimed in claim 1, wherein a promoter used for expression of the gene is a promoter of a glyceraldehyde-3-phosphate dehydrogenase gene (GLD), acid phosphatase gene (PHO5) or uridine-galactose diphosphate-4-epimerase gene (Gal10).
- 10 4. Yeast as claimed in claim 1, wherein the yeast is Saccharomyces cerevisiae NA74-3A (ρ^-), Saccharomyces cerevisiae K33-7B (ρ^-), Saccharomyces cerevisiae NA87-11A (ρ^-) or Saccharomyces cerevisiae NAX-50D (ρ^-).
5. A method for preparing a protein foreign to yeast, which comprises culturing respiratory-deficient yeast excluding Saccharomyces cerevisiae AH22R⁻, said respiratory-deficient yeast being transformed with a DNA containing a gene encoding a protein foreign to yeast, and producing and accumulating the
- 15 protein in a culture.
6. A method for preparing a protein as claimed in claim 5, wherein the protein is human lysozyme or human EGF.
7. Yeast as claimed in claim 1, which is Saccharomyces cerevisiae NA74-3A(ρ^-)/pTFL710T.
8. Yeast as claimed in claim 1, which is Saccharomyces cerevisiae NA74-3A(ρ^-)/pPFL725T.
- 20 9. Yeast as claimed in claim 1, which is Saccharomyces cerevisiae NA74-3A(ρ^-)/pGFE213.
10. Yeast as claimed in claim 1, which is Saccharomyces cerevisiae NAX-50D(ρ)/pTFL710T:

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FIG. 1

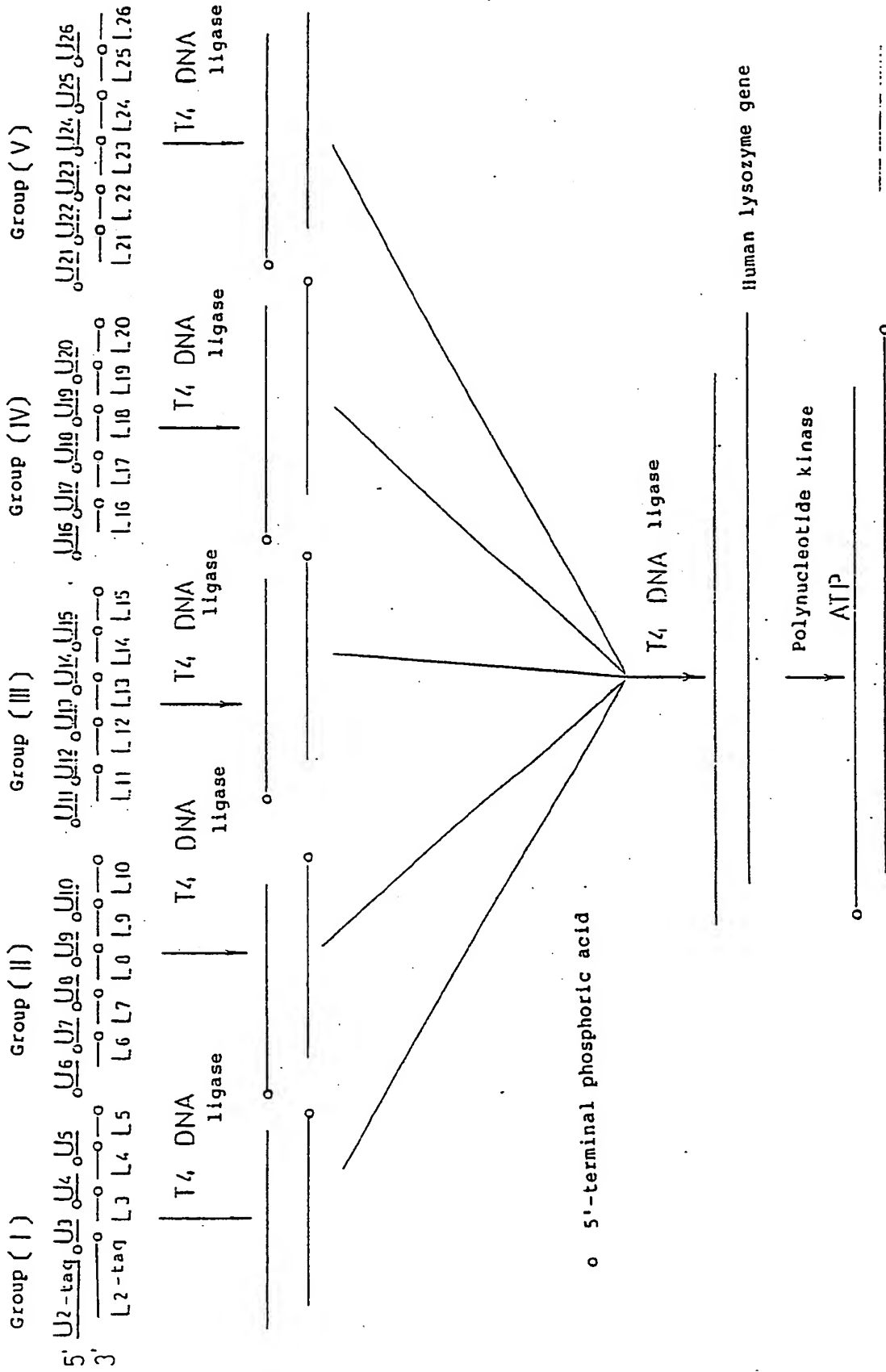


FIG. 2

Taq I

TCGAGAGATGCGAATTAGCCAGAACTTTGAAGAGATTGGGTATGGACGGCTACCGTGGTATTTC
 AGCTCTACGCTTAATCGGTCTTGAACTTCTCTAACCCATACCTGCCGATGGCACCAATAAG

Hpa II

Mae IAlu I

TTTAGCCAACTGGATGTCTTGCTAAGTGGGAATCCGGGCTATAACACTAGAGCTACCAATTAC
 AAATCGGTTGACCTACACAGAACGATTCAACCCTTAGGCCGATATTGTGATCTCGATGGTTAATG

Xba I

AACGCTGGCGACCGTTCTACAGACTATGGTATTTTCCAAATTAACTCTAGATATTGGTGTAAACG
 TTGGACCGCTGGCAAGATGTCTGATACCATAAAGGTTTAATTGAGATCTATAACCATTGC

Alu I

ATGGCAAGACTCCAGGTGCCGTCAACGCCCTGTCACCTTATCTTGCTCAGCTTTGCTTCAGGACAA
 TACCGTTCTGAGGTCCACGGCAGTTGCGGACAGTGAA TAGAACGAGTCGAAACGAAGTCCCTGTT

Hha I

CATTGCTGATGCTGTTGCCCTGGCGCTAAGAGAGTTGTCCGTGACCCACAGGGTATTAGAGCCTGG
 GTAACGACTACGACAACGGACGCGATTCTCTCAACAGGCACTGGGTGTCCCATAAATCTCGGACC

GTCGCTTGGAGAAACAGATGCCCAAAATAGAGATGTCAGACAAATACGTTCAAGGTTGTGGTGT
 CAGCGAACCTCTTTGTCTACGGTTTATCTCTACAGTCTGTTATGCAAGTTCCAAACACCAACAA

Xho I

Alu I

AATAGCTCGA
 TTATCGAGCT

FIG. 3

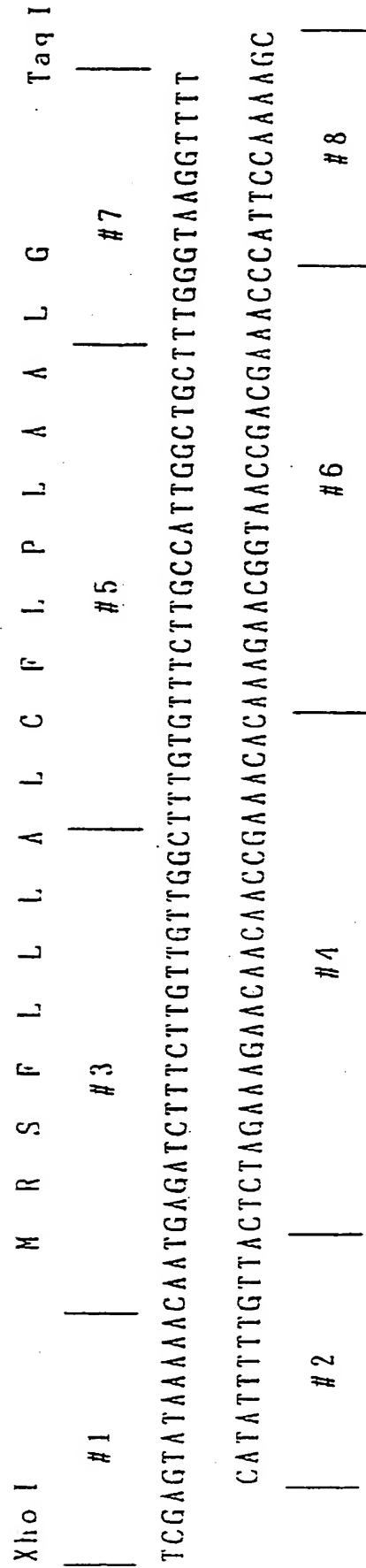


FIG. 4

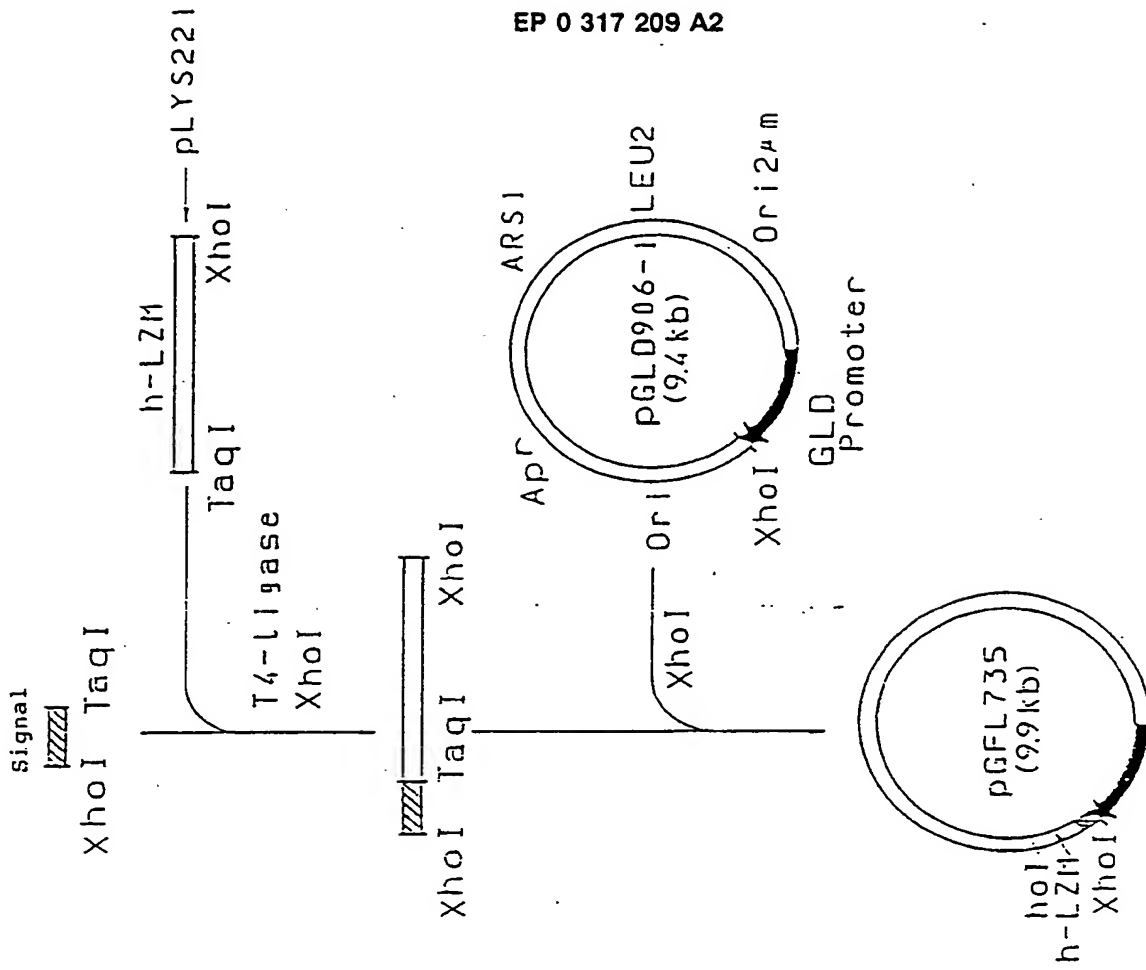


FIG. 7-1

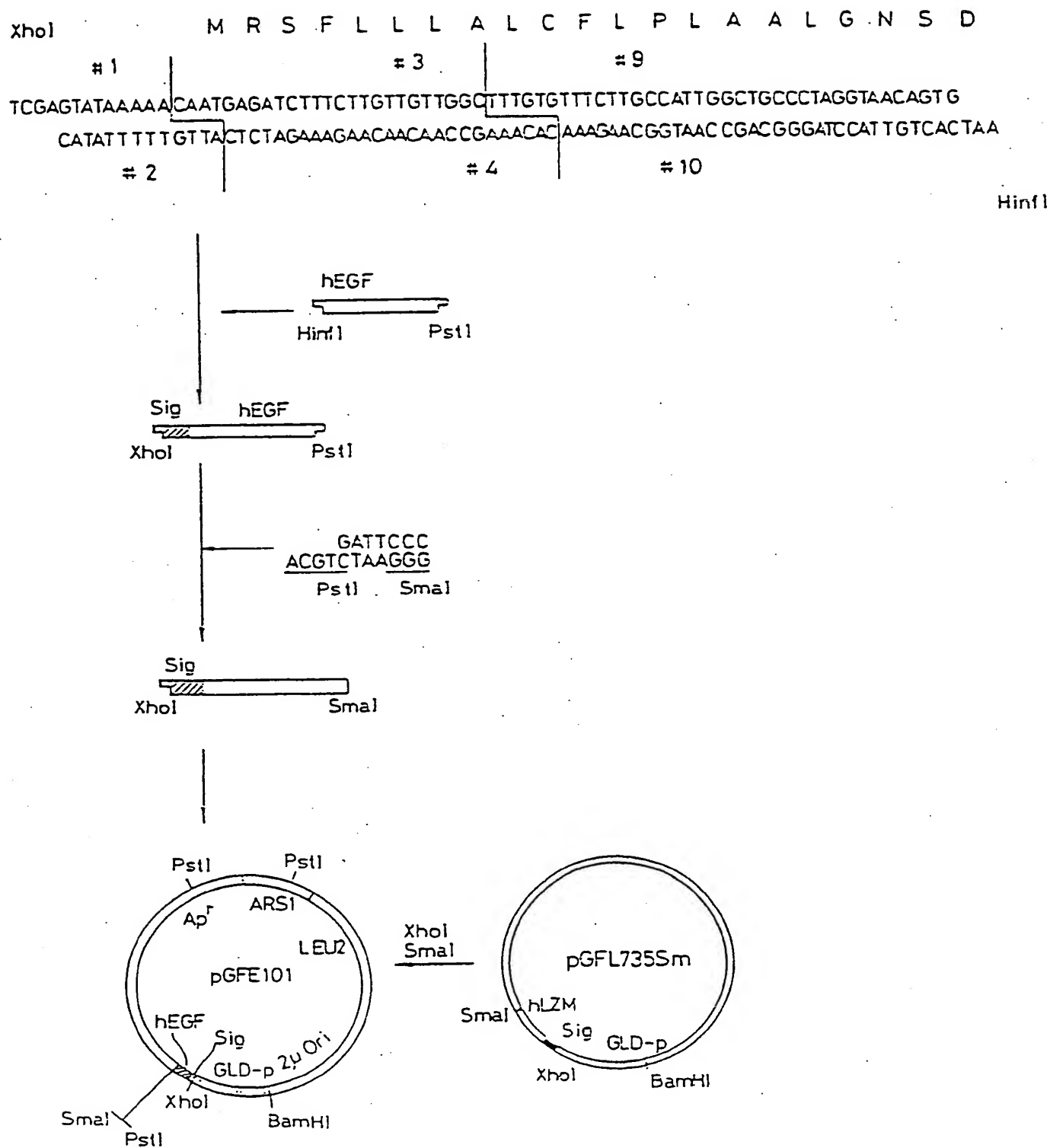


FIG. 7 - 2

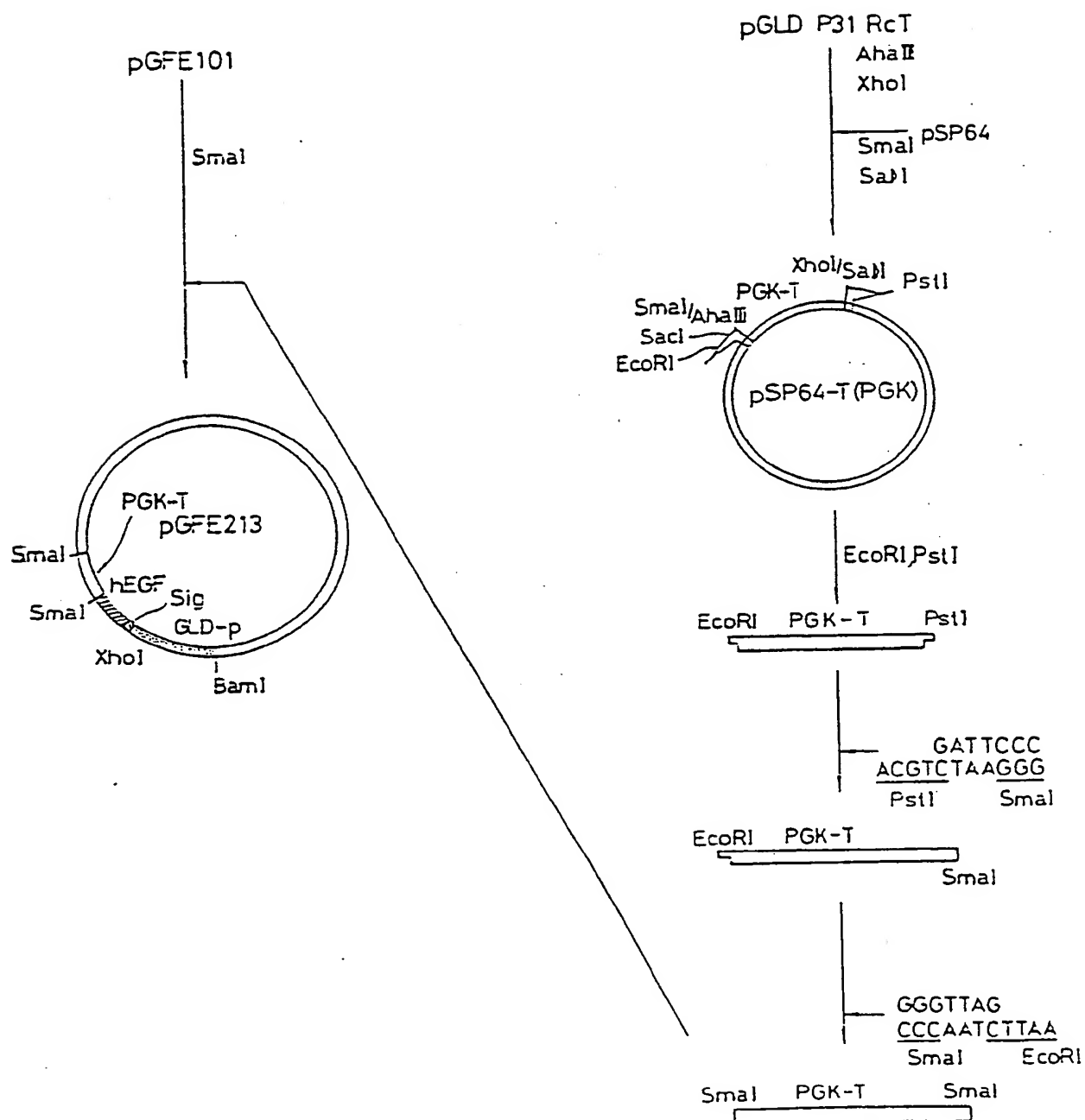


FIG. 8

5' GATCACCGCGGATCCGGTTACCGTCGACTATAATGACAGATC 3'
TGGCGCCTAGGCCAATGGCAGCTGATATTACTGTCTAGAGCT